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Stimulating Influence of Cerebral Ganglia on *in vitro* Incorporation of Tritiated Leucine into Ovaries of *Eisenia fetida* Sav. (Annelida: Oligochaeta)

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ABSTRACT—In sexually active *Eisenia fetida*, gametogeneses are controlled by a cerebral neurohormone. Present results show that the central nervous system (CNS, *i.e.* cerebral ganglia, the circum-oesophageal ring and the ventral nerve cord cut at clitellum level) stimulates the uptake of ^{3}H-leucine by ovaries. Cultured together with CNS, ovaries incorporate twice as much ^{3}H-leucine as when cultured alone. This protein synthesis is independent of the duration of the culture of explants, of their period of incubation with a precursor and of the concentration of this precursor. However, in culture *in vitro*, ovaries syntheses are low as the percentage of incorporation goes from 0.11% in isolated ovaries to 0.22% in ovaries associated with CNS. This confirms that in ovarian oocytes vitellogenesis is low. Among the different ganglia of CNS, the cerebral ganaglia alone stimultate the incorporation of ^{3}H-leucine with ovaries.

It is suggested that a same cerebral neurohormone is thought to control cephalic regeneration, clitellogenesis, testis differentiation and ovarian protein metabolism.

INTRODUCTION

During genital activity, the testicular tissues of *Eisenia fetida* secrete an androgen under the control of a neurohormone produced by the central nervous system (C.N.S.) [1, 2]. This hormone is synthesized by the cerebral ganglia [3, 4] which also induce the male and female gametogenetic activity in *Eisenia fetida* [5, 6] and in *Allolobophora icterica* [7]. Moreover, they control, by inhibition, the cephalic regeneration of *E. fetida* [8] and are necessary to the maintaining of clitellum turgescence in mature worms [9]. Several endocrine actions can therefore be attributed to cerebral ganglia in worms of the lumbricid oligochaetes [10]. A cerebral hormone controlling cephalic regeneration and clitellum turgescence has been isolated in *E. fetida* and partly purified [11–14]. It is a peptidic substance [15] of a molecular weight between 1500 and 2000 [12]. In this species, a purified fraction from the brain stimulates the secretion of testicular androgen [16].

But, previous experiments of organotypic culture had shown that the survival time of a gonad associated to C.N.S. was always longer than that of a gonad cultured on its own. Besides its neurohormonal control of gametogeneses, the C.N.S. also seems to exert a sort of "trophic" effect on metabolism in gonad (testis or ovaries).

Before studying the ways the control mechanisms of gonad function we felt inclined to separate the different parameters. In order to achieve this goal, a simple biological test had first to be defined for the metabolic activity of gonads. The ovary, which does not require the presence of cerebral hormone for its differentiation, was chosen. A low vitellogenesis was found in growing oocytes, the vitelline material being composed of

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protein granules together with glycogen particles and fat droplets [17]. This test measured the uptake of tritiated leucine by ovaries of *E. fetida*, cultured on their own or in accociation with C.N.S. (or different ganglia of C.N.S.).

MATERIALS AND METHODS

Experiments were done on sexually active *Eisenia fetida* Sav. Each culture, composed of 3 ovaries isolated or associated with 3 C.N.S. (i.e. cerebral ganglia, the circum-oesophageal nerve ring and the ventral nerve cord cut at the clitellum level), lasted 4 or 8 days. After culture, explants were incubated in a physiological medium with a radioactive amino-acid, leucine, for 1, 2 or 4 hr. When the ovaries and C.N.S were associated, they would be incubated together or separately. The controls were composed of 3 ovaries associated with 3 muscle fragments and cultured in the same experimental condition. Ovaries and muscle fragments were then incubated together or separately with leucine. The quantity of muscle was roughly equal to that of a central nervous system. The culture technique and the medium composition have been previously descrived [18]. The physiological incubating medium was the Holtfreter solution at 8.77‰ (saline solution at 8.13‰ NaCl). This solution is isotonic with the coelomic fluid of *E. fetida*. The tritiated leucine (L-leucine ^{3}H, 3–4–5; specific activity 5550 GBq/mM; CEA, France) was added to the medium in the proportion of 370, 740 or 1480 kBq/ml of medium. Incubations were done in a Kotterman agitator for 2 hr at 20°C in eppendorf tubes containing 100 µl of solution. After incubation, explants were rinsed in Holtfreter physiological solution, in 3 successive baths of 10 min each at least to wash away the unincorporated leucine. They were then sonicated in the Holtfreter solution and treated in 20% trichloracetic acid to precipitate the labelled proteins. Total hydrolysis of explants was done in 200 µl of Soluene (Packard). Five ml of scintillating liquid (insta-gel) was added to each sample. Radioactivity was measured by scintillating liquid spectrophotometry in an Intertechnique SL 300 counter. In order to take into account the results of the first series, the subsequent experiments needed 4 to 8 days of culture followed by 1, 2 or 4 hr incubation in a medium with a ^{3}H-Leu concentration of 1110 kBq/ml of physiological solution.

In order to localize the origin of the C.N.S. activity, 3 ganglia of different levels (cerebral ganglia, C.G., sub-oesophageal ganglia, S.O.G., and ganglia of male genital segments, G.G. o and female G.G.o) were cultured with 3 ovaries for 4 days. In the end, each association was incubated for 1 hr in a tritiated leucine solution (1110 kBq/ml), then treated according to the conditions previously described made to measure radioactivity.

The values given by the radioactivity counter were normalized to 1 mg of wet weight for each sample and expressed in counts per minute (cpm). Before we weighed the explants. The average values obtained after weighing 10 organs were: C.N.S. (=C.G.+S.O.G.+ganglia of the first 13 segments)=1.3 mg; C.G.=0.2 mg; S.O.G.=G.G.o=G.G.o=0.1 mg; ovaries=0.1 mg; muscle fragment=1.3 mg. The mean of radioactivity measures on several explants was calculated for each experimental series together with standard deviation. Student's t-test was used to compare the different mean values. Results are shown in tables by + or − indicating that, in comparisons, the differences are significant or not for $p<0.01$.

RESULTS

A. Measure of *in vitro* incorporation of ^{3}H-leucine with ovaries associated or not with C.N.S. Comparison with controls (ovaries+muscles)

1. *Explants cultured for 8 days, then incubated for 2 hr in tritiated leucine (concentrations 370, 740 or 1480 kBq/ml)*

Results are presented in Table 1 and in Figures 1 and 2. The radioactivity of ovaries associated to C.N.S. (incubated together or separately, columns 2 and 3) was always superior to that of isolated ovaries (col. 1) at whatever concentration of ^{3}H-Leu (370 to 1480 kBq/ml). Indeed, for these different concentrations, radioactivity increased from 21200 to 66400 cpm/mg of wet weight isolated ovaries (col. 1) while it reached from 56000 to 178000 cpm/mg of wet weight of ovaries incubated with C.N.S. (col. 2) and from 52000 to 230000

TABLE 1. *In vitro* incorporation of ^{3}H-Leu at different concentrations with ovaries of *E. fetida* associated or not to central nervous system (controls: ovaries associated to muscle). Duration of culture: 8 days; incubation period: 2 hr. Results in cpm/mg wet weight

		Ovaries and CNS associated in culture			Ovaries and muscles associated in culture		
	Ovaries	incubated together	incubated separately		incubated together	incubated separately	
			ovaries	CNS		ovaries	Muscles
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
370 kBq/ml	21200 ± 1000	56000 ± 150	52000 ± 100	4100 ± 10	27700 ± 100	25000 ± 1000	350 ± 40
	Comparisons with (1)	+	+		−	−	
740 kBq/ml	56200 ±14000	74000 ± 500	54000 ±11000	4300 ± 850	69800 ± 1800	67000 ± 4300	1800 ± 300
	Comparisons with (1)	+	−		−	−	
81480 kBq/ml	66400 ±18000	17800 ± 1000	232000 ± 150	18100 ± 100	133700 ± 3000	118700 ± 5400	1700 ± 400
	Comparisons with (1)	+	+		+	+	
	Comparisons with (2)				+	+	
	Comparisons with (2)				+	+	
	Comparisons with (3)				+	+	

t-test: + = significant difference for $p<0.01$; − = not significant difference.

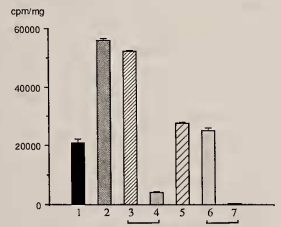

FIG. 1. *In vitro* incorporation of ^{3}H-Leu ovaries of *Eisenia fetida* associated or not to C.N.S. Duration of culture: 8d; incubation period: 2 hr in a physiological medium with ^{3}H-Leu (370 kBq/ml). 1: isolated ovaries. 2: ovaries and C.N.S. 3 and 4: ovaries and C.N.S. associated together and incubated separately (3: ovaries, 4: C.N.S.). 5: ovaries and muscle associated and incubated together. 6 and 7: ovaries associated to muscle and incubated separately (6: ovaries, 7: muscles). Ordinate: radioactivity expressed in cpm/mg wet weight.

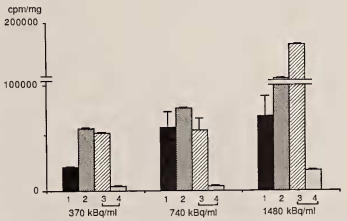

FIG. 2. *In vitro* incorporation of ^{3}H-Leu ovaries of *Eisenia fetida* associated or not to C.N.S. Duration of culture: 8 days; incubation period: 2 hr in a medium with ^{3}H-Leu at different concentrations (370, 740 or 1480 kBq/ml). For legend see Fig. 1.

cpm/mg of ovaries incubated independently of C.N.S. (col. 3). The comparison with results obtained in ovaries cultured on their own showed that difference were always significant ($p<0.01$). It should be noted that in this experimental series (col. 3 and 4) the radioactivity measured in C.N.S. was 12 times below that measured in ovaries: the uptake of radioactive precursor by C.N.S. was small. So C.N.S. stimulate the uptake of ^{3}H-Leu by ovaries *in vitro* for an amino-acid concentration varying from 370 to 1480 kBq/ml of incubating medium. When ovaries cultured without C.N.S. were incubated with tritiated leucine at a concentration of 370 kBq/ml (i.e. 2×10^6 cpm in 100 μl solution), the radioactivity measured in an ovary of 0.1 mg was 2120 cpm, which corresponds to a low rate of uptake of tritiated leucine (0.11%). Spontaneous protein synthesis by ovaries were very low. In the presence of C.N.S., these protein syntheses correspond to an incorporation rate of 0.26% (col. 3) and of 0.28% (col. 2). Even under the influence of C.N.S., syntheses were still very low. For the same tritiated leucine concentrations, radioactivity in the controls (ovaries cultured and incubated with muscle fragments, col. 5) varied from 27200 to 133700 cpm/mg wet weight. When ovaries and muscles were incubated separately (col. 6) the counting revealed, according to concentration, an increase in radioactivity of 25000 to 118700 cpm/mg wet weight of ovaries and of 350 to 1700 cpm/mg wet weight of muscles. It can be observed that ^{3}H-Leu of 350 to 1700 cpm/mg wet weight of muscles. It can be observed that ^{3}H-leucine incorporation by muscle is very low and can be overlooked. Another conclusion is that muscle has no stimulating effect on the incorporation of this amino-acid with ovaries *in vitro*. Indeed, the radioactivity measured in control ovaries (col. 5 and 6) was almost always equal to that in isolated ovaries (col. 1). The differences observed are not significant, except in the experimental series in which the concentration of tritiated leucine is 1480 kBq/ml: the radioactivity of ovary-muscle associations (col. 5 and 6) represented twice (133700 and 118700 cpm/mg) that of isolated ovaries (66400 cpm/mg, col. 1). Nevertheless the Student's t-test revealed that the averages obtained in columns 5 and 6 differed significantly ($p<0.05$) from those found in column 2 as well as in column 3. Therefore, even in this case, it can be said that muscle has no effect over ovary in culture; statistics confirm that muscle is a good control. Nevertheless the remarkable increase of incorporation by the ovary in this experimental series cannot be explained.

In later experiments, the control series, ovaries and muscle incubated separately (col. 6 and 7) will be suppressed. Indeed, in such experimental conditions, the radioactivity specific to muscle represents less than 3% of the total radioactivity and the sum of the radioactivities of ovaries and muscles is approximately equal to that measured in the ovary-muscle association (col. 5).

From the results of this experimental series, a protocole was chosen from the later series: concentration in ^{3}H-Leu, 1110 kBq/ml of medium; duration of cultures, 4 and 8 days; incubation period with a radioactive precursor, 1, 2, and 4 hr.

2. *Explants cultured for 4 or 8 days, then incubated 1, 2, or 4 hours in a medium with tritiated leucine (concentration 1110 kBq/ml)*

Culture and incubation time vary in this experimental series. Results are summed up in Table 2 and Figures 3 and 4. After 4 or 8 days in culture, explants are incubated 1, 2 or 4 hr in a physiological medium with ^{3}H-Leu (1110 kBq/ml). The radioactivity measured in explants (ovaries and C.N.S. incubated together, col. 3 and 4, or separately, col 5 and 6) is always superior to that found in isolated ovaries (col. 1 and 2). So when ovaries cultured separately for 4 days are incubated 1 to 4 hr in tritiated leucine, the radioactivity goes from 11400 to 46600 cpm/mg wet weight of ovaries (col. 1). In the same conditions, ovaries associated to C.N.S. reveal a radioactivity respectively 2 to 5 times greater, whether incubated together (col. 3) or separately (col. 5). Statistically, the differences are highly significant ($p<0.01$) and similar to those obtained after 8 days in culture (col. 2, 4, and 6).

It can therefore be concluded that C.N.S. has a stimulating effect on the incorporation of tritiated leucine with ovaries *in vitro*, when explants cultured 4 or 8 days are then incubated for 1 to 4 hr with this amino-acid. It should be noted, without satisfactory explanation, that explants cultured for

TABLE 2. *In vitro* incorporation of ^{3}H-Leu (1110 kBq/ml) with ovaries of *Eisenia fetida* associated or not to central nervous system (controls: ovaries associated to muscle). Duration of culture: 4 or 8 days; incubation period: 1, 2 or 4 hr. Results in cpm/mg wet weight

		Isolated ovaries		Ovaries+CNS associated in culture						Ovaries+muscles associated in culture and incubated together	
				incubated together		incubated separately					
						ovaries		CNS			
		4d	8d	4d	8d	4d	8d	4d	8d	4d	8d
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1 hr		11400 ± 2600	24300 ± 7800	55900 ±20000	43400 ±12000	50500 ± 2000	38200 ±19000	2800 ± 80	3400 ± 1200	13600 ± 5800	19300 ± 4600
	Compa-	with (1)		+		+				−	
	risons	with (2)			+		+				−
2hr		36800 ± 8700	16400 ± 6200	56800 ±16700	40200 ± 3300	50400 ± 3800	49500 ± 7800	5200 ± 100	4200 ± 1800	21200 ± 7000	28200 ± 9700
	Compa-	with (1)		+		+				−	
	risons	with (2)			+		+				−
4 hr		46600 ±13000	68700 ±23000	79000 ±20000	71500 ±10600	79600 ±42000	97300 ±25000	8400 ± 1600	13800 ± 4000	43000 ±15000	51500 ±20000
	compa-	with (1)		+		+				−	
	risons	with (2)			+		+				−

t-test: +=significant difference for $p<0.01$; −=not significant difference.

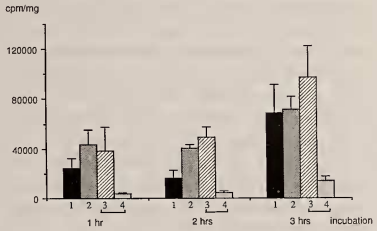

FIG. 3. *In vitro* incorporation of ^{3}H-Leu ovaries of *Eisenia fetida* associated or not to C.N.S. Duration of culture: 8 days; incubation period of 1, 2 or 4 hr (1110 kBq/ml of medium). For legend see Fig. 1.

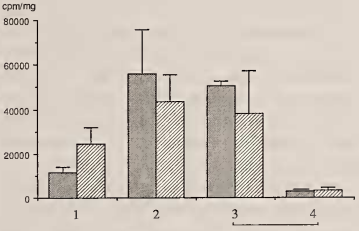

FIG. 4. *In vitro* incorporation of ^{3}H-Leu ovaries of *Eisenia fetida* associated or not to C.N.S. Duration of culture: 4 or 8 days; incubation period: 1 hr (1110 kBq/ml). Stippled columns: 4 days in culture; striped columns: 8 days in culture. For legend see Fig. 1.

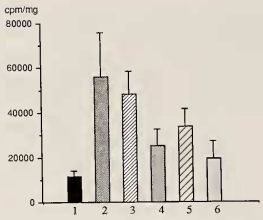

Fig. 5. *In vitro* incorporation of ^{3}H-Leu (1110 kBq/ml) by ovaries of *E. fetida* associated or not to different ganglia of C.N.S. In associations, ovaries and ganglia were incubated together. Duration of culture: 4 days; incubation period: 1 hr. 1: isolated ovaries; 2: ovaries+C.N.S.; 3: ovaries+C.G.; 4 ovaries+S.O.G.; 5: ovaries+G.G.o; 6: ovaries+G.G.o. Ordinate: radioactivity expressed in cpm/mg wet weight.

8 days do not incorporate a quantity of leucine strictly proportional to the incubation period.

On the other hand, in experimental series (col. 5 to 8) where ovaries are incubated independently of C.N.S. The incorporation of tritiated leucine by C.N.S. (col. 7 and 8) is low and clearly below (7 to 18 times) that measured in ovaries. It only represents 5 to 12% of the radioactivity of the ovaries and C.N.S. These results are comparable to those present in the previous paragraph (Table 1).

When ovaries and muscles, associated in culture (controls), are incubated together for 1, 2, or 4 hr with tritiated leucine (concentration: 1110 kBq/ml), radioactivity varies from 13600 to 43000 cpm/mg for 4 days in culture (col. 9) and from 19300 to 51500 cpm/mg for 8 days in culture (col. 10). Comparatively the radioactivity measured in isolated ovaries (col. 1 and 2) goes from 11400 to 46600 cpm/mg for 4 days in culture and from 24300 to 68700 cpm/mg for 8 days.

The analysis of previous series (Table 1, col. 7) having shown that the incorporation of tritiated leucine by muscles was negligible, the experiments recorded in Table 2, col. 9 and 10, take into account the ovary-muscle association. Considering that the differences between the mean values of the series 1 and 2, on the one hand, and 9 and 10 on the other hand are not significant, it can be concluded that muscles have no effect on the incorporation of tritiated leucine with ovaries *in vitro*. These results thoroughly confirm those of previous series (paragraph 1) i.e. muscle is an excellent control. The homogeneity of results enable the selection of a culture duration of 4 days and an incubation period of 1 hr for all subsequent experiments. Moreover, the incorporation specific to C.N.S., on average less that 10% than the total incorporation, can be overlooked and the values which were considered would only concern ovaries +C.N.S. incubated together.

B. Measure of incorporation of tritiated leucine with ovaries *in vitro* associated to different genglia of C.N.S.

The results presented in the Table 3, correspond to the following experimental conditions: 4 days in culture followed by 1 hr incubation in a physiological medium with ^{3}H-Leu (1110 kBq/ml). The radioactivities measured in association of ovaries with different parts of C.N.S. (C.G., S.O.G., G.G.o or G.G.o) are respectively of 48200, 25200, 33700 and 19200 cpm/mg wet weight (col. 3 to 6). These values should be compared to the radioactivity previously measured in isolated ovaries (11400 cpm/mg, col. 1) and in ovaries incubated with C.N.S. (55900 cpm/mg, col. 2) in identical experimental conditions. Only the radioactivity of ovary-C.G. associations (col. 3) is almost equal to that of ovaries cultured with C.N.S. (the difference between these values are not significant). On the other hand, it is clearly above that of isolated ovaries (col. 1) (difference highly significant). Besides, the difference between the values of radioactivity of ovaries associated to C.G. (col. 3) or to C.N.S. (col. 2) and that of ovaries associated to other nervous ganglia (S.O.G., col. 4; G.G.o, col. 5; G.G.o, col. 6) is equally significant. From these results we can conclude that in C.N.S., the C.G. alone stimulate the *in vitro* incorporation of tritiated leucine with ovaries.

On the contrary, it is difficult to explain the small difference of radioactivity of ovary-G.G.o. associations (col. 6) in relation to that of ovaries+G.G.o (col. 5) or S.O.G. (col. 4). The Student's t-test reveals that values do not differ significantly

TABLE 3. *In vitro* incorporation of ^{3}H-Leu (1110 kBq/ml) with ovaries of *Eisenia fetida* associated or not to different ganglia of central nevous system. Duration of culture: 4 days, incubation period: 1 hr. Results in cpm/mg wet weight

	Isolated ovaries (1)	Ovaries+CNS (2)	Ovaries+CG (3)	Ovaries+SOG (4)	Ovaries+GGo (5)	Ovaries+GGo (6)
	11400 ± 2600	55900 ±20000	48200 ±10500	25200 ± 7500	33700 ± 8000	19200 ± 8000
Comparisons	with (1)	+	+	+	+	−
	with (2)		−	+	+	+
	with (3)			+	+	+
	with (4)					−
	with (5)					−

t-test: + = significant difference for $p<0.01$; − = not significant difference.

between them. Similary, the difference between the radioactivity measured in ovaries + G.G.o (col. 6) and that of isolated ovaries (col. 1) is not significant. Finally, the difference between radioactivity values of these isolated ovaries and those of ovaries associated to C.G. (col. 3), S.O.G. (col. 4) or G.G.o (col. 5) is significant.

DISCUSSION

The present results clearly show the stimulating action of the central nervous system over the *in vitro* incorporation of tritiated leucine with ovaries. Indeed, the radioactivity measured in ovaries cultured with C.N.S. is about twice as much as that found in isolated ovaries, whatever the duration of culture (4 or 8 days), the incubation period (1, 2 or 4 hr) and the precursor concentration (370, 740 or 1480 kBq/ml of medium). However, the ovarian protein syntheses are low, as percentages of incorportion are about 0.27%, at the most, in presence of C.N.S. and about 0.11% for isolated ovaries. This confirms all cytological and cytochemical observations: ovarian oocytes reveal a low vitellogenesis because eggs of *Eisenia* are poor in yolk [19]. It is also interesting to note that the *in vitro* incorporation of ^{3}H-Leu with C.N.S. is very low and always below that of ovaries. Finally, in the same experimental conditions, the muscles represent an insignificant radioactivity which can be overlooked; they have no stimulating effect on the incorporation of leucine by ovaries.

Among the different ganglia of C.N.S., only the cerebral ganglia exert some influence over the protein metabolism of ovaries. The responsible substance seems distributed according to a gradient of activity, from the C.G. to the ganglia of the 10th and 11th segment (testes level): it could diffuse within the C.N.S. from the C.G. which is the elaboration site. This would seem to confirm that the radioactivity measured in associations ovaries + S.O.G., ovaries + G.G.o or G.G.o is still quite important. Distantly acting v ovaries (in the 13th segment) and mostly through the circulatory system, this substance could be akin to a neurohormone. This anteroposterior gradient in C.N.S. could be related to that experimentally established for the inhibition of cephalic regeneration [20]. Such a similar distribution could lead to presume that the trophic action revealed here, would be due to the same cerebral neurohormone, already well known in cephalic regeneration, male genital differentiation and clitellogenesis. However, before attempting to verify this hypothesis, it is necessary to confirm present results. For this purpose, an autoradiographic study is in process, to define the site of protein syntheses in the ovary.

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